Bacillus clausii MB9 from the east coast regions of India: Isolation, biochemical characterization and antimicrobial potentials

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Ninety-one bacterial colonies were isolated from water samples of five coastal regions of southern India (Tamil Nadu). All these isolates were culture-purified and screened for antimicrobial activity against a battery of human pathogens. Among these, 33% of the isolates ($n = 30$) exhibited strong bactericidal and fungicidal properties against pathogens. One promising strain, designated as MB9, with strong antimicrobial activity against most of the pathogens tested was selected for further studies. Morphological, cultural, physiological, biochemical properties and molecular characterization, 16S rRNA sequencing and BLAST analysis (IMTECH, Chandigarh) indicated this strain as *Bacillus clausii* (100% similarity with GenBank sequences). The secondary metabolite (culture supernatant) produced by *B. clausii* MB9 at different salinities, temperatures and pH values was found to be stable at elevated temperature conditions. Moreover, the culture supernatant from this novel isolate remains active and retains its antimicrobial activities even after the supernatant was subjected to autoclaving temperatures (121°C). There was no significant loss of antifungal activity after treatment with various detergents. The results obtained in the present investigation indicate that *B. clausii* MB9 produced a novel, highly thermostable secondary metabolite showing a wide spectrum of antimicrobial activities. Studies relating to the use this isolate as probiotics and also for the production of biocontrol agents (insect and mosquito larvicides), industrial enzymes (for leather dehairing and as a component of detergent) and molecular characterization of the active principle in the supernatant are in progress.

Keywords: Antagonistic activity, *Bacillus clausii*, microorganisms, secondary metabolites.

THE biota of marine microorganisms has developed unique metabolic and physiological functions that not only ensure survival in extreme habitats, but also offer a potential for the production of novel enzymes for potential exploitation. Out of the large number of species examined, only a fraction of marine bacteria have been isolated and cultured. Among them, alkaliphilic *Bacillus* strains are of considerable importance in biotechnological applications[1–3]. Almost all *Bacillus* species identified produced metabolites and exhibited antimicrobial activities against a broad spectrum of bacteria, including *Bacillus* sp. involved in food spoilage and pathogenic organisms such as *Clostridium perfringens*, *Staphylococcus aureus* and *Listeria monocytogens*[4]. During the past two decades, marine bacteria have highlighted the tremendous potential of these microorganisms as a source of new bioactive secondary metabolites[5,6]. There is growing awareness of the need for development of new antimicrobial agents for the treatment of human, animal and plant diseases. No reports documenting the presence of alkaliphilic *Bacillus* sp. from the Indian soil and coastal environments are available in the literature. However, novel *B. lehensis* (MLB2 (T)) was reported recently from Leh region, Jammu and Kashmir[7] and *B. licheniformis* SPT27, a producer of extracellular alpha amylase was isolated from the alkaline soil of the eastern coastal region of Cambay, Gujarat[8]. However, in an extensive survey of microbial diversity at marine salterns near Bhavnagar, Gujarat, no *Bacillus* sp. was documented[9].

In this article, we report the characterization of *B. clausii* MB9 recently isolated from the eastern coastal environment of Tamil Nadu (South India). The present study focuses on the production of secondary metabolite from this isolate with tremendous antimicrobial and industrial potential. Our results provide insights into the wide spectrum antimicrobial ability of the identified *Bacillus* species from the Indian coastal environment.

Materials and methods

Isolation of organisms from coastal waters

Ninety-one bacterial isolates from coastal water samples at Mandapam (79.12′E, 9.28′N), Rameswaram (79.29′E, 9.15′N), Thondi (79.04′E, 9.45′N), Thiruchendur (78.11′E, 8.08′N) and Thoothukudi (78.13′E, 8.45′N), Tamil Nadu,

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India were studied. Samples were collected in sterile plastic containers according to microbiological procedures and shifted to the laboratory for further analysis. All the bacterial colonies were isolated in marine agar (Himedia), purified and screened for antimicrobial activity against a battery of human pathogenic microorganisms.

Screening of marine bacteria for antagonistic activity

Primary and secondary screening: A total of 91 bacterial strains isolated (marine Agar, Himedia) from the coastal water samples were screened for the production of antibacterial substance. In the primary screening, antimicrobial activity was assessed against the target pathogenic microorganisms[10]. From the pure cultures, marine bacterial strains were spotted on target organisms swabbed in Muller Hinton Agar (MHA) plates. Based on the zone of inhibition, each bacterial strain was selected for secondary screening and further analysis.

Secondary screening was done by agar well diffusion assay[11] for testing the antagonistic activities of marine bacterial isolates. Selected isolates were grown on Starch Casein Broth (SCB) at 35°C in a rotary shaker (200 rpm) for 1–4 weeks and the samples were pelleted down every week. Then the culture broth was centrifuged at 9500 *g* for 15 min and the culture supernatant collected and filtered through bacterial filter (Millipore filter; 0.45 µm) to obtain the cell-free extract. The wells were cut using a sterile cork of 8 mm diameter and 100 µl of supernatant was loaded into each well for the assay of antagonistic activity by well diffusion assay against different pathogens (Table 1). The results were recorded after incubation at 35°C for 24 h.

Identification of isolates by IMTECH

The isolate MB9 was identified originally as a strain of actinomycete by our laboratory based on morphological characteristics. Subsequently the isolate was submitted to Microbial Type Culture Collection (MTCC) and GeneBank, IMTECH, Chandigarh (a nodal agency involved in microbial type culture collections and testing) for characterization. 16S rRNA sequencing was performed and compared with sequences of *Bacillus* sp. available in the databank. The sequence analysis for 16S rRNA and phylogenetic comparisons was performed by IMTECH.

Optimization for secondary metabolite production

Culture optimization was performed for the isolate MB9 in SCB at different pH values (4 and 9), NaCl concentrations (1, 3, 5, 7 and 9%) and incubated for 4 weeks at

Table 1. Antimicrobial activity of supernatants of *Bacillus clausii* (MB9) cultured in different temperatures, pH, salinities and after heat treatment

	Zone of inhibition (mm)											
	Temperature (°C)				pH		Salinity (%)					Heat-treated
Target organism	25	35	45	55	4	9	1	3	5	7	9	121°C
Bacillus subtilis – NCIM 2063	T	22	12	T	T	14	18	20	14	12	–	20
Escherichia coli – NCIM 2871	T	18	T	–	–	14	16	20	–	12	12	20
Enterobacter aerogenes – MTCC 111	T	22	14	10	14	21	12	22	16	14	–	18
Klebsiella pneumoniae – NCIM 2957	14	16	16	–	16	14	12	16	–	12	16	16
Micrococcus luteus – NCIM 2871	12	20	14	16	12	12	18	20	12	T	–	22
Proteus vulgaris – NCIM 2813	14	20	16	20	16	12	18	24	18	T	14	20
Pseudomonas aeruginosa – NCIM 2200	T	14	14	–	18	–	14	14	12	12	–	16
Salmonella typhi – MTCC 734	T	16	12	11	12	20	20	18	20	24	T	14
Serratia marcescens – MTCC 97	T	18	12	18	12	16	14	18	16	T	T	16
Shigella flexneri – MTCC 1457	–	22	12	11	12	14	16	22	12	T	T	20
Staphylococcus aureus – NCIM 2079	–	16	14	12	–	14	14	16	18	16	14	20
Staphylococcus epidermidis – NCIM 2493	T	22	12	T	14	24	18	22	18	14	18	20
Streptococcus mutans – MTCC 890	12	16	14	T	12	16	12	16	18	14	12	20
Vibrio cholerae – MTCC 1738	12	18	12	11	12	22	12	18	12	12	T	18
Vibrio parahaemolyticus – MTCC 451	–	22	12	14	12	14	12	24	–	T	–	14
Aspergillus niger – NCIM 586	28	30	16	T	16	–	12	28	–	T	14	16
Candida albicans – MTCC 8471	12	16	16	T	12	22	16	16	–	18	T	14
Microsporum gypseum – MTCC 2830	10	16	10	T	10	11	12	16	10	12	T	12
Penicillium oxalicum – MTCC 4931	12	18	12	T	14	–	24	18	12	T	12	14
Trichophyton rubrum – MTCC 296	12	12	10	T	12	12	10	12	12	12	T	14

Culture supernatant concentration is 100 µl. Each datapoint is the mean of three experiments.
–, Negative; T, Trace; MTCC, Microbial Type Culture Collections, IMTECH, Chandigarh; NCIM, National Collection of Industrial Microorganisms, Pune, Maharashtra.

35°C and at different temperatures (25°C, 45°C and 55°C) in replicates. The culture supernatant was separated by centrifugation at 9500 *g* for 15 min and used for antimicrobial studies. Based on the highest antimicrobial potential, the culture supernatant obtained at 35°C was selected and subjected to high temperature (121°C) by autoclaving in glass containers for 15 min and used for antimicrobial studies[12].

Effect of shelf-life on antimicrobial activity

The shelf-life (storage time) on antimicrobial activity of the culture supernatant of MB9 was determined by storing the supernatant at 4°C in ampoules for different time periods (1, 2, 4, 6, 8 and 12 months). After the specified period, 100 µl from each tube was added to wells of MHA plates already swabbed with test organisms[12].

Effect of detergents

The effect of detergents on the antimicrobial activity was determined by adding the culture supernatant with different detergents like SDS (sodium dodecyl sulphate), Tween-20, Tween-80, Cetrimide and Triton X-100 and incubating them at 30°C for 6 h. Detergents were dissolved in distilled water at a concentration of 0.01 g/ml. Next 100 µl of the supernatant was mixed with 100 µl of detergent[13] and incubated at 35°C for 24 h. Detergents added to distilled water were used as control. Agar well diffusion assay was performed against test organisms using the above extracts along with positive and negative control, as explained earlier.

Screening for proteolytic activity

Bacillus clausii MB9 was screened on skim milk agar medium containing skim milk powder 10% (w/v), peptone 0.5% (w/v) and agar 2% (w/v) at pH 8.0. Formation of the clear zone of lysis was recorded[14]. The proteolytic activity was recorded depending on the zone of clearance and growth of the organism.

Protease production

The culture medium used in this study for protease production contained 0.5% glucose (w/v), 0.75% peptone (w/v), 0.5% (w/v) $MgSO_4 \cdot 7H_2O$, 0.5% (w/v) KH_2PO_4, and 0.01% (w/v) $FeSO_4 \cdot 7H_2O$ maintained at 37°C for 24–72 h in a shaking incubator (140 rpm). At the end of each fermentation period, the whole fermentation broth was centrifuged at 9500 *g* at 4°C for 15 min and the clear supernatant was used as crude enzyme preparation. Next, 1 ml of supernatant was added to 1 ml of 1 M NaOH and absorbance was read at 440 nm. One unit (U) of protease activity is defined as the micromole of substrate converted per minute under standard assay conditions. The protein content was determined according to Lowry *et al.*[15] using bovine serum albumin as standard.

Spectrophotometer and Fourier transform infrared spectroscopy

UV spectra were recorded using UV/VIS Spectrophotometer (Hitachi). NEXUS – 672 and 8400S Shimadzu MODEL Fourier transform infrared spectroscopy (Japan) were used for analysis of the 12 culture supernatants. The spectrum was taken in the mid IR region of 400–4000 cm^{-1}. The spectrum was recorded using ATR (attenuated total reflectance) technique. The sample was directly placed in the sodium chloride crystal and the spectrum recorded in the transmittance mode.

NMR analysis

Bruker (300 MHz) NMR spectroscopy was used for analysis of the 12 culture supernatants. The samples were dissolved using deuterated chloroform ($CDCl_3$) as solvent.

Results

Antagonistic activities – Primary and secondary screening

Out of 91 bacterial isolates screened in the preliminary study, 58 (64%) were selected for secondary screening (Figures 1 and 2). These isolates were able to exert an inhibitory effect against at least one of the target organisms tested. Among the 58 isolates, a novel strain of *B. clausii* MB9 revealed the efficiency of broad spectrum antimicrobial activity. Hence, the strain MB9 isolated from the coastal region of Rameswaram was selected for further studies (Table 1; Figure 3).

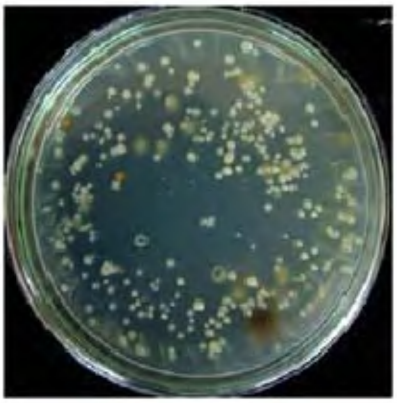

Figure 1. Isolation of marine bacteria.

Identification of B. clausii

Morphological studies revealed that the isolate MB9 was non-pigmented, circular, rhizoid, slightly raised and shiny with smooth colonies. The growing cells were Gram-positive, facultatively aerobic, motile, sporulating, rod-shaped (3–4 μm × ≈1.0 μm) organisms with peritrichous flagella. Spores were ellipsoidal and positioned centrally in non-swollen sporangium. No bacterial growth was observed at pH 4.0 and weak growth was seen at pH 6.0 in the liquid and solid media used. The isolates grew well in nutrient broth at a pH range of 8–9 and showed salt tolerance at NaCl concentrations up to 10% (w/v), indicating that they are obligate alkalophile. Bacterial growth was observed in the temperature range from 20°C to 52°C, with an optimum around 42°C. The isolate was positive for the utilization of lactose, hydrolysis of gelatin and casein, enzyme tests of catalase, oxidase and reduced nitrate. Negative results were observed for growth in MacConkey agar, tests for indole, methyl red and Voges Proskauer, utilization of citrate, formation of hydrogen sulphide, hydrolysis of casein, urea, Tween-20, -40 and -80 and activities of arginine dihydrolase, ornithine decarboxylase and lysine decarboxylase (Table 2).

Partial sequencing of 16S rRNA was performed by MTCC, IMTECH, Chandigarh. BLAST analyses of the sequence data revealed 100% identity with *B. clausii*, when the sequences were compared with *B. clausii* and *Bacillus* species related sequences available in the databases (Figure 4). MB9 was confirmed as *B. clausii* based

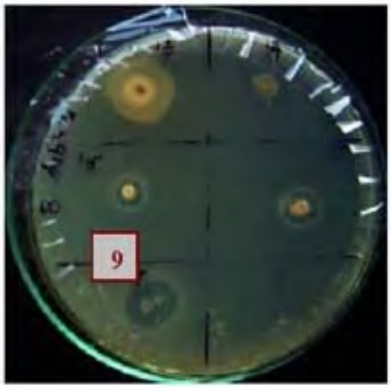

Figure 2. Primary screening – antimicrobial activity.

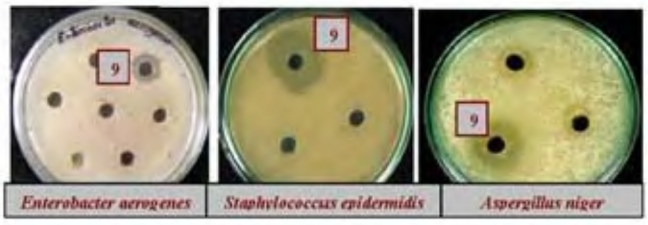

Figure 3. Antimicrobial activity of *Bacillus clausii* MB9 against Gram-negative, Gram-positive and fungal organisms.

Table 2. Morphological and biochemical tests for identification of MB9

Test	MB9
Colony morphology	
Configuration	Circular
Margin	Rhizoid
Elevation	Slightly raised
Surface	Shiny and smooth
Pigment	–
Opacity	Translucent
Gram's reaction	Positive
Cell shape	Rods
Size (μm)	Length: 3–4 μm, width: ≈1.0 μm
Arrangement	Singles
Spores (s)	Sporulating
Endospore	+
Position	Central
Shape	Ellipsoidal
Sporangia bulging	–
Motility	+
Physiological tests	
Growth at temperature (°C)	
8	–
15	–
20	+
37	+
42	+
52	+
Growth in NaCl (%)	
2.0	+
5.0	+
7.0	+
10.0	W
Growth at pH	
4.0	–
5.0	–
6.0	W
8.0	+
9.0	+
Growth under anaerobic condition	+
Biochemical test	
Growth in Mac Conkey Agar	–
Indole test	–
Methyl red test	–
Voges Proskauer test	–
Citrate utilization	–
H_2S production	–
Casein hydrolysis	–
Esculin hydrolysis	+
Gelatin hydrolysis	+
Starch hydrolysis	W
Urea hydrolysis	–
Catalase test	+
Oxidase test	+
Nitrate reduction	+
Arginine dihydrolase	–
Ornithine decarboxylase	–
Lysine decarboxylase	–
Tween-40 hydrolysis	–
Tween-60 hydrolysis	–
Tween-80 hydrolysis	–
Acid from glucose	–
Acid from lactose	+
Gas from glucose	W
O/F test	–

W, Weak; +, positive; –, negative.

GGCCTTCGGGTCGTAAAGCTCTGTTGTGAGGGAAGAAGCGGTACCGTTCGAATAGGGCGGTACCTTGACGGT
ACCTCACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGG
AATTATTGGGCGTAAAGCGCGCGCAGGCGGCTTCTTAAGTCTGATGTGAAATCTCGGGGCTCAACCCCGAGC
GGCCATTGGAAACTGGGGAGCTTGAGTGCAGAAGAGGAGAGTGGAATTCCACGTGTAGCGGTGAAATGCGTA
GAGATGTGGAGGAACACCAGTGGCGAAGGCGACTCTCTGGTCTGTAACTGACGCTGAGGCGCGAAAGCGTGG
GGAGCAAACA

Figure 4. Partial sequence analysis of 16S rRNA of isolate MB9 by BLAST (370 bp). Sequence analysis revealed a score of 733 bits and an identity of 100% for 32 sequences available in the databank.

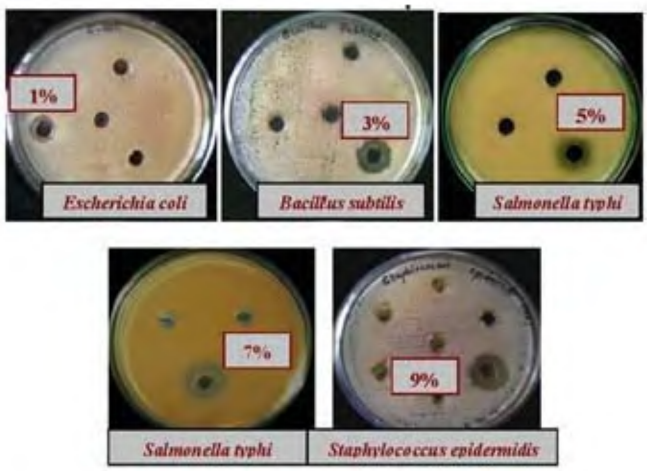

Figure 5. Effect of salinity on antimicrobial activity of *B. clausii* MB9. Other marine bacteria were tested with MB9. However, only MB9 is shown here.

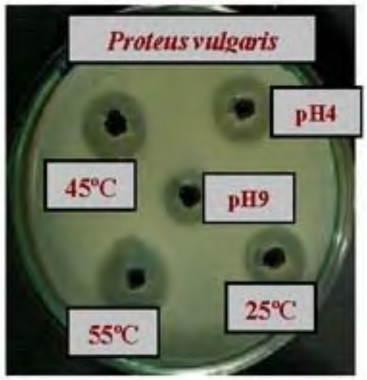

Figure 6. Effect of temperature and pH on antimicrobial activity of *B. clausii* MB9.

on morphological, physiological and biochemical characteristics and also by 16S rRNA sequence analysis. The organism submitted to IMTECH was given a GenBank accession number (MTCC-8326) and deposited as type strain.

Culture optimization and antimicrobial activities

Results showed the sensitivity ranging from 12 to 18 mm zone of inhibition in all the organisms tested, except *Escherichia coli* and *Staphylococcus aureus* at highly acidic pH of 4 (90%). Even under basic culture conditions (pH 9), the supernatant showed zone of inhibition against 85% of the test organisms. Supernatants raised from cultures in different salinities (1, 3, 5 and 7%) showed significant antagonistic activity, except at 9%. The isolate MB9 at 25°C, 35°C, 45°C and 55°C showed significant zone of inhibition to 50%, 100%, 95% and 45% of the test organisms respectively (Table 1; Figures 5–7). Thus the isolate MB9 produces culture supernatant with maximum antimicrobial property at 35°C and 45°C (i.e. 100 and 95% of all pathogens tested showed zone of inhibition).

Effect of temperature

The stability of the supernatant was tested by heat treatment (by autoclaving). The results indicated that all the test organisms showed sensitivity ranging from 12 to 22 mm zone of inhibition to heat-treated culture supernatant of *B. clausii* MB9. The supernatant of MB9 showed elevated antagonistic activity against 20% of the tested organisms when compared to the supernatant not subjected to heat treatment (Table 1; Figures 7 and 8).

Effect of shelf-life

The shelf-life of supernatant from MB9 isolate showed an increased zone of inhibition from 22 to 26, 14 to 24 and 30 to 34 mm zone of inhibition against *Bacillus subtilis*, *Pseudomonas aeruginosa* and *Aspergillus niger* respectively (Table 3; Figure 9). Thus the supernatant from MB9 retained its antibacterial activity even after 12 months of storage.

Effect of detergents

The supernatant of *B. clausii* MB9 exhibited zone of inhibition against most of the test organisms. However, the activity of the supernatant was moderately affected by detergents such as SDS, Tween-20, Cetrimide and Triton-X-100, showing reduced zone of inhibition when compared to that without detergents. However, there was no significant loss of antimicrobial activity even after the treatment with detergents, as indicated by the antimicrobial activity studies (Table 3; Figure 9).

Table 3. Effect of shelf-life and detergents on antimicrobial activity of *B. clausii* MB9 supernatant

Shelf-life of supernatant (months)	Zone of inhibition (mm)	Detergents with supernatant	Zone of inhibition (mm)
Against (G^-) *Pseudomonas aeruginosa*		Against (G^-) *P. aeruginosa*	
1	14	SDS	14
2	16	Tween-20	14
4	16	Tween-80	12
6	16	Cetrimide	14
8	18	Triton X-100	12
12	20	Supernatant (only)	16
Against (G^+) *Bacillus subtilis*		Against (G^+) *B. subtilis*	
1	22	SDS	18
2	24	Tween-20	16
4	24	Tween-80	14
6	24	Cetrimide	14
8	24	Triton X-100	16
12	24	Supernatant (only)	16
Against fungi *Aspergillus niger*		Against fungi *A. niger*	
1	30	SDS	22
2	32	Tween-20	22
4	30	Tween-80	24
6	34	Cetrimide	24
8	34	Triton X-100	23
12	34	Supernatant (only)	24

*Culture supernatant volume is 100 µl. Each datapoint is the mean of three experiments.

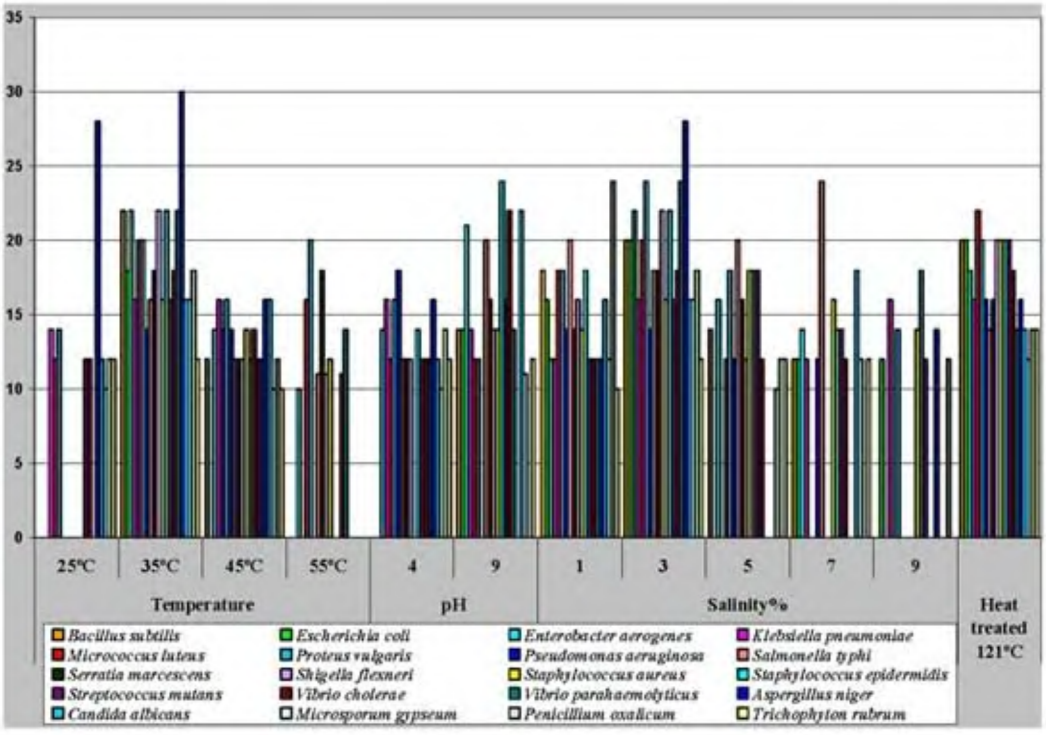

Figure 7. Antimicrobial activity of supernatant of *B. clausii* (MB9) cultured in different temperatures, pH values, salinities and heat inactivation.

Proteolytic activity and protein estimation

The proteolytic activity was assayed using skim milk agar and expressed as diameter of clear zone (in mm). *B. licheniformis* MB2 exhibited the highest proteolytic activity showing a clear zone of lysis with a diameter of 45 mm followed by *B. clausii* MB9 with a diameter of 43 mm (Figure 10). The amount of protein present in the

culture supernatant was determined using BSA standard and the *B. clausii* MB9 exhibited the highest protein content of 140 µg/l.

UV, IR and NMR spectral analysis

The UV spectral data exhibited strong absorption (λ-max) at 354 nm for *B. clausii* MB9. The FTIR spectrum exhibited absorption bands mostly indicating probable presence of groups of aliphatic amines and amines [1122.61; 1250.88 – aliphatic amines – (C–N)]. ^{1}H NMR (300 MHz) spectrum of the samples in deuterated chloroform ($CDCl_3$) showed major peaks between the region of 9 and 15 δ (ppm), indicating the presence of compounds with amine group, i.e. ($R_2C{=}N{-}NH_2$) compounds (Figure 11).

Figure 8. Effect of heat treatment on supernatant of *B. clausii* MB9. Other marine bacteria were tested with MB9. However, only MB9 is shown here.

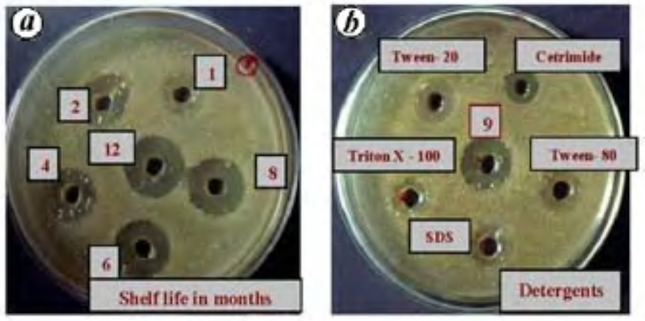

Figure 9. Effect of shelf-life and detergents on antagonistic activity of supernatant of *B. clausii* MB9. ***a***, Against (G^+) *Bacillus subtilis*. ***b***, Against (G^-) *Pseudomonas aeruginosa* (9, Supernatant of MB9 at 35°C).

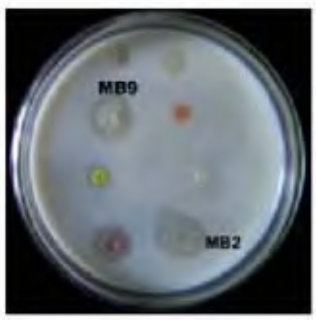

Figure 10. Proteolytic activity of supernatant *B. clausii* (MB9) on skim milk agar.

Discussion

In 1947, Rosenfeld and Zobell[16] had carried out the first detailed study of antibiotic-producing marine bacteria. Since then, there are several reports of antibiotic-producing marine bacteria showing the antagonistic effect against human pathogens[17–19], as strains of pathogenic bacteria that recently emerged are unresponsive or multidrug resistant to the already discovered antibiotics that are in use.

In the present study two isolates from the coastal region of Rameswaram were identified as *B. clausii* (our laboratory reference – MB9; MTCC-8326) and *B. licheniformis* (MB2; MTCC-8310) with haloalkaliphilic culture characteristics. *Bacillus* spp. have been described traditionally as aerobic saprophytic soil microorganisms. Many members of the *Bacillus* group continue to be dominant bacterial workhorses in microbial fermentation for the production of novel proteins[20]. A handful of studies were undertaken to classify the truely alkaliphilic *Bacillus* strains and alkali-tolerant *Bacillus* strains that are phylogenetically distinct, with the exception of *B. alcalophilus* and *B. cohni*[1,21,22]. Recent studies, however, report the widespread presence of *B. clausii* from different environmental setting[4,23–28].

The *B. clausii* MB9 grew well in nutrient broth in the pH range 8–9 and showed salt tolerance up to 10% (w/v), NaCl, indicating that the isolate is an obligate alkaliphilic. Although 73 bacterial isolates were identified in an extensive survey of microbial diversity at marine salterns near Bhavanagar, Gujarat, no *Bacillus* sp. has been documented[9]. In the marine environment, 90% of bacteria are Gram-negative with different characteristics[29] and the Gram-negative cell wall is better adapted for survival in the marine environment[30]. However, in our study the *Bacillus* spp. reported were Gram-positive, indicating that their origin could be due to terrestrial run-off from rivers, as reported widely for marine actinomycetes[31].

In the present study, the culture supernatant from *B. clausii* MB9 showed potent and wide-spectrum antimicrobial activity. At a culture condition of 35°C, the MB9 isolate produced supernatant showing antimicrobial activity against all the organisms tested (both Gram-positive and Gram-negative bacteria and fungi). The results revealed a high degree of antimicrobial activity (zone of inhibition more than or equal to 20 mm) towards *B. subtilis*, *Enterobactar aerogens*, *Micrococcus luteus*, *Proteus vulgaris*, *Shigella flexneri*, *Staphylococcus epidermidis*, *Vibrio parahaemolyticus* and *A. niger* (Table 3; Figure 3). Several *Bacillus* isolates demonstrated antagonistic activity against a broad range of bacterial strains[32]. *B. subtilis* isolate 259 (from broilers) presented the largest antibacte-

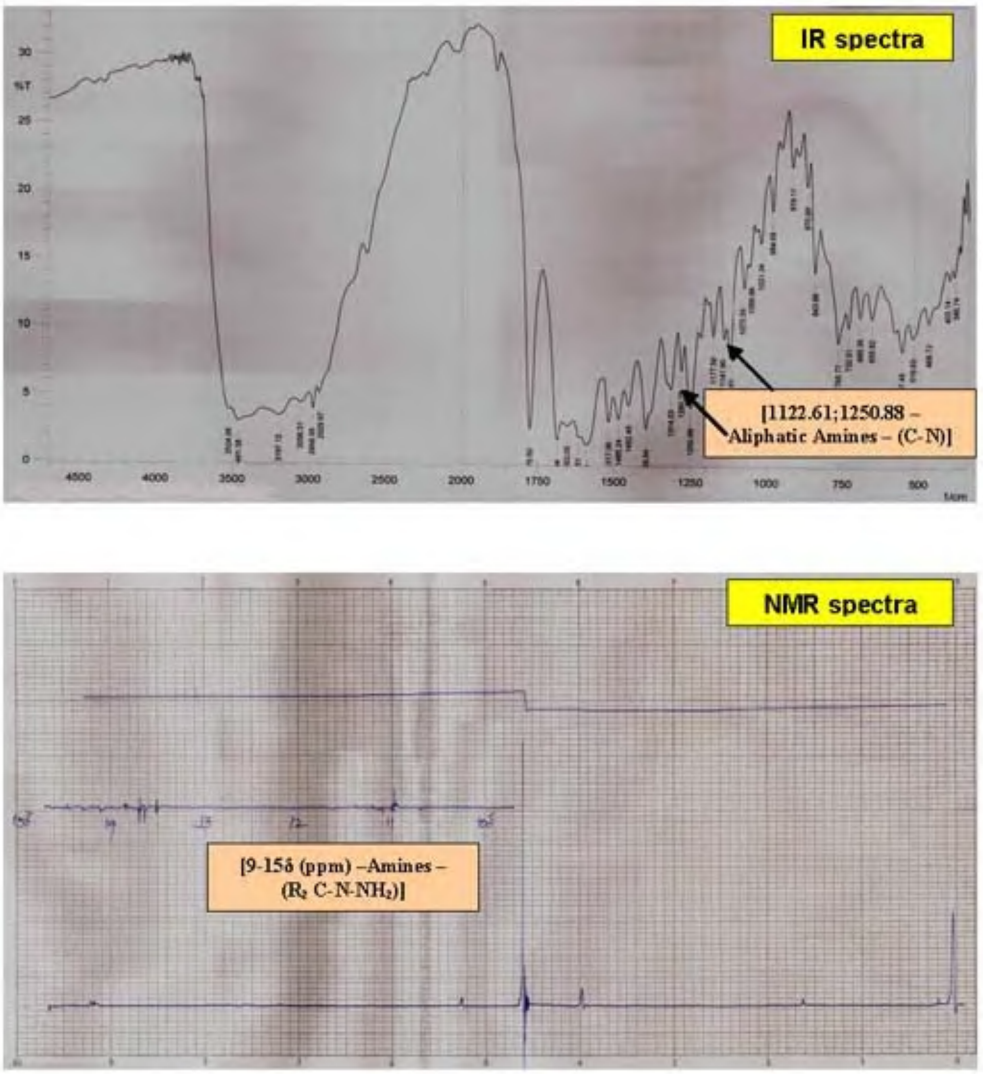

Figure 11. IR and NMR spectra of compounds from *B. clausii* MB9.

Table 4. Proteolytic activity of various *Bacillus* organisms and their optimum pH and temperature

Organism	Optimal pH	Temperature (°C)	Reference
Bacillus sp. RGR14	11.0	60	38
B. subtilis	7–12	15–65	39
Bacillus sp. SSR1	10.0	40	40
B. pumilus	10.0	55	41
B. licheniformis MIR29	12.0	60	42
B. horicoshi	9.0	45	43
B. brevis	10.5	37	44
B. clausii GMBAE42	11.0	60	26
Bacillus sp.	11.0	70	45, 46
Bacillus sp. P-2	9.6	90	47
B. clausii I–52	9.0	60	25
B. clausii	9.0	up to 80	27

rial spectrum, exhibiting inhibitory activity against several Gram-positive species such as *Clostridium perfringens*, *Enterococcus faecalis*, *Staphylococcus aureus* and different *Bacillus* species[4,32]. Competitive exclusion of pathogens by the production of bacteriocins by *Bacillus* sp. has been reported[33]. Also, *in vitro* the anti-*Helicobacter pylori* activity of probiotic strain of *B. subtilis* was shown to be dependent on the production of antibiotics[34].

In the present study, *B. clausii* MB9 produced supernatant with potent antimicrobial activity in varying salinity range (salt concentrations). However, at a salinity of 3%, the supernatant showed significant antagonistic activity against almost all the organisms tested. With culture temperatures of 25°C and 35°C and at 3% salinity, maximum antagonistic activity was observed against the fungi *A. niger*.

The shelf-life studies of the supernatant of *B. clausii* MB9 revealed an increased zone of inhibition against *P. aeruginosa* than the fresh supernatant. This observation needs to be explored further to understand the molecular and biochemical mechanisms behind the effect of shelf-life on the activity of supernatant against human pathogens, such as *P. aeruginosa*. Shelf-life-dependent

increase of antimicrobial activity was also noticed against *A. niger* in the present study. Similar observations on the antifungal metabolites produced by *Streptomyces albidoflavus* stable at various storage time durations has been reported[35]. The extended shelf-life of *Bacillus* sp. over a wide range of temperatures, salinities and pH values has been reported[4]. There are several reports in the literature regarding the alkaline proteases produced by alkalophilic bacilli (Table 4).

As presented in Table 4, the current study has also revealed proteolytic activity at pH 9 and 60°C. The highest zone of inhibition was recorded against *M. luteus* after heat treatment (121°C) of the supernatant from isolate MB9. Levels equivalent to this antagonistic activity were also observed against many pathogens, viz. *B. subtilis*. *E. coli*, *P. vulgaris*, *S. flexneri*, *Staph. aureus*, *Staph. epidermidis*, *Strept. mutans*, *E. aerogens* and *V. cholerae*. Such heat stable property of *Bacillus*-derived supernatants has not been found in the literature.

Jaruchoktaweechai *et al.*[36] reported that the isolation, antibacterial activity and structures of macrolactins from the marine *Bacillus* sp. Sc026, including the known compound, macrolactin F. Nitrogenated compounds are dominated by amines and amides. Kelecom[37] reported that among 145 isolates of marine microorganisms, 29.6% has been reported to produce amines and amides. All these previous data lend support to our present finding of nitrogenated compounds dominated by amines in the culture supernatant of *B. clausii*.

Our data presented the highest zone of inhibition against *M. luteus* after heat treatment of the supernatant. This novel strain of *B. clausii* MB9 with supernatant showing high degree of thermal stability opens new insights into the future applications of these industrially important strains for the production of a new class of antibiotics, detergents and other products to be used in industrial processes.

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